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CD4-CD8 Lineage Commitment Is Regulated by a Silencer Element at the ThPOK Transcription-Factor Locus

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SUMMARY

The transcription factor ThPOK is necessary and sufficient to trigger adoption of the CD4 lymphocyte fate. Here we investigate the regulation of ThPOK expression and its subsequent control of $CD4^+$ T cell commitment. Treatment of immature thymocytes with anti-TCR (T cell receptor) showed that TCR signals were important in ThPOK induction and that the $CD4^+8^{lo}$ stage was the likely target of the inductive TCR signal. We identified at the ThPOK locus a key distal regulatory element (DRE) that mediated its differential expression in class I- versus II-restricted $CD4^+8^{lo}$ thymocytes. The DRE was both necessary for suppression of ThPOK expression in class I-restricted thymocytes and sufficient for its induction in class II-restricted thymocytes. Mutagenesis analysis defined an essential 80bp core DRE sequence and its potential regulatory motifs. We propose a silencer-dependent model of lineage choice, whereby inactivation of the DRE silencer by a strong TCR signal leads to CD4 commitment, whereas continued silencer activity leads to CD8 commitment.

INTRODUCTION

Development of αβ T cells in the thymus proceeds through three major stages defined by the expression pattern of the coreceptor molecules CD4 and CD8, i.e., $CD4^-CD8^-$ (double negative or DN), $CD4^+CD8^+$ (double positive or DP), and $CD4^+CD8^-$ or $CD4^-CD8^+$ (single positive or SP). During the DP to SP transition, most thymocytes pass through an intermediate $CD4^+8^{lo}$ stage, irrespective of whether they are destined to become SP CD4 or CD8 cells (Guidos et al., 1990; Kydd et al., 1995; Lucas and Germain, 1996; Lundberg et al., 1995; Suzuki et al., 1995; Barthlott et al., 1997; Chan et al., 1998). The αβTCR complex is first expressed at the DP stage, allowing its engagement by intrathymic peptide-MHC ligands. TCR signaling can induce two different processes in developing thymocytes, i.e., negative selection leading to death by apoptosis, or positive selection leading to thymocyte activation and differentiation into SP T cells.

Coincident with positive selection, thymocytes undergo alternate commitment to either the cytotoxic or the helper T cell lineages, generating class I-restricted SP $CD8^+$ and class II-restricted SP $CD4^+$ thymocytes. The mechanism by which this correlation between MHC restriction and functional phenotype is achieved has remained controversial. Initially, it was suggested either that lineage commitment might be directed by qualitatively distinct signals initiated upon TCR and coreceptor engagement by class I- or II MHC ligands (instructive model) (Robey et al., 1991), or that it occurred randomly and was followed by a selection step that eliminates thymocytes that express the inappropriate coreceptor (stochastic-selective model) (Chan et al., 1993; Davis et al., 1993). However, experiments to test these hypotheses gave inconsistent outcomes, suggesting that the models needed to be refined (Robey et al., 1991; Borgulya et al., 1991; Chan et al., 1994; Matechak et al., 1996; Robey et al., 1994). A quantitative-instructive model instead proposes that stronger and weaker TCR signals, respectively, promote CD4 and CD8 commitment (Matechak et al., 1996). This model is based on the fact that the cytoplasmic tail of CD4 binds the critical signaling factor Lck with substantially higher affinity than that of CD8α (Ravichandran and Burakoff, 1994; Veillette et al., 1988). Studies in which TCR signaling is modulated quantitatively have lent strong support to this model (Hernandez-Hoyos et al., 2000; Legname et al., 2000; Liu and Bosselut, 2004). It has been suggested that lineage commitment is not completed until the intermediate $CD4^+8^{lo}$ stage, because many of these cells are not yet irreversibly committed to a particular lineage (Bosselut et al., 2003; Brugnera et al., 2000). Indeed, the distinct kinetic-signaling model postulates that lineage commitment is determined at the $CD4^+8^{lo}$ stage, on the basis of the relative ability of coreceptors to contribute to TCR signaling (Singer and Bosselut, 2004). Because CD8 is selectively downmodulated in $CD4^+8^{lo}$ thymocytes, TCR signaling will be specifically impaired for class I-restricted thymocytes at this stage. The intracellular pathway downstream of Lck and Zap70 that regulates alternate lineage commitment remains unknown. Although a preferential role for the Ras-Mek-Erk pathway in CD4 development has been suggested (Bommhardt et al., 1999; Sharp et al., 1997), genetic approaches do not support this (Alberola-Ila and Hernandez-Hoyos, 2003; Fischer et al., 2005).

Some insight into the intracellular control of lineage commitment was provided by the discovery of HD mice, which carry a spontaneous mutation that causes class II-restricted thymocytes

to mature into SP CD8 rather than CD4 T cells (Davé et al., 1998; Keefe et al., 1999). In light of the quantitative-instructive model, such redirection might reflect diminished TCR signaling capacity. However, all biochemical assays of TCR signaling proved normal, and the efficiency of positive selection, as assessed by the proportions of SP thymocytes, and negative selection, both processes that are highly sensitive to alterations in TCR signaling, are unaffected (Keefe et al., 1999; He et al., 2005). These data argue strongly that the HD mutation does not affect the initiation of the CD4 commitment signal, but rather the downstream interpretation of this signal. The helper T cell-deficient (HD) phenotype further implies that lineage commitment and positive selection are at some level mechanistically distinct, because the HD mutation affects only the former process. Given that both processes appear to be controlled by TCR signaling, this implies divergence and specialization in signaling pathways downstream of the TCR with a specific pathway leading to CD4 commitment. The HD mutation has been mapped to the locus encoding the Zn-finger transcription factor ThPOK, also known as Zbtb7b, Zfp67, or cKrox (He et al., 2005; Kappes et al., 2006). The expression pattern of ThPOK during thymic development is tightly regulated in a manner that indicates a specific role in CD4 commitment. Thus, ThPOK RNA is first detected at the $CD4^+8^{lo}$ stage, and ThPOK mRNA expression is substantially higher in class II- than class I-restricted cells at this stage, consistent with preferential induction by class II ligands (He et al., 2005). Constitutive expression of ThPOK during thymic development causes redirection of class I-restricted thymocytes to the CD4 lineage. Thus, ThPOK expression is both necessary and sufficient for CD4 commitment. Importantly, neither overexpression nor inactivation of ThPOK is sufficient to drive development to the SP stage in the absence of a positive selection signal, consistent with the view that ThPOK does not mimic or modulate TCR signaling.

Several additional transcription factors play selective roles in CD4 or CD8 development and might therefore be involved in lineage commitment, notably Gata-3 and Runx3. Constitutive expression of Gata-3 specifically blocks CD8 development, whereas its conditional deletion blocks CD4 development (Hernandez-Hoyos et al., 2003; Nawijn et al., 2001; Pai et al., 2003). In neither case are affected thymocytes redirected to the opposite lineage. Hence, Gata-3 is necessary but not sufficient for CD4 development. Runx factors clearly play an important role in regulating the CD8 development program, including the CD8 lineage-specific suppression of CD4 (Sawada et al., 1994; Siu et al., 1994; Taniuchi et al., 2002; Sato et al., 2005; Ehlers et al., 2003; Egawa et al., 2007). However, a direct role of Runx factors in initiating lineage commitment appears unlikely on the basis of results from constitutive Runx3 transgenic mice. Thus, although development of SP CD4 thymocytes is impaired in Runx3 transgenic mice, this is corrected by constitutive CD4 expression (Grueter et al., 2005). CD4 deficiency is known to cause redirection of class II-restricted thymocytes to the CD8 lineage, presumably by weakening or interrupting TCR signaling (Matechak et al., 1996; Tyznik et al., 2004). Hence the primary effect of Runx factors on lineage commitment appears to lie in controlling CD4 expression.

As outlined above, the intracellular pathways that control alternate CD4 or CD8 lineage choice remain poorly understood. The finding that lineage commitment is controlled by differential expression of ThPOK provides a central access point for unraveling these pathways. In particular, through the determination of how differential expression of ThPOK is controlled, it should ultimately prove possible to identify the upstream pathways that control lineage commitment. Accordingly, in the present study we examined two aspects of ThPOK regulation, i.e., its upstream control by TCR signaling and its immediate control by *cis*-acting regulatory elements. This study led to two important insights. First, we provide direct evidence that TCR signaling is an important contributor to ThPOK induction. Second, we demonstrate that differential ThPOK expression is controlled by a transcriptional silencer element. We propose a model whereby CD4 commitment requires inactivation of the silencer, whereas continued activity of the silencer leads to CD8 commitment.

RESULTS

Regulation of ThPOK by TCR Signaling

ThPOK mRNA amounts are markedly higher in class II- than class I-restricted $CD4^+8^{lo}$ cells, suggesting that ThPOK induction is regulated instructively by relative TCR signal strength (He et al., 2005; Sun et al., 2005). However, in vitro anti-TCR treatment of uncommitted DP thymocytes failed to induce ThPOK mRNA (data not shown). Reasoning that additional in vivo stimuli might be required, we carried out anti-TCR cross-linking in vivo. For this purpose, we administered anti-TCRβ antibody to MHC class II-deficient ($IAb^{-/-}$) mice whose thymocytes can only undergo positive selection by class I ligands and normally express little ThPOK. Antibody treatment led to strong induction of ThPOK transcription at the $CD4^+8^{lo}$ stage by 48 hr (Figure 1A). This was accompanied by the appearance of SP CD4 thymocytes, as previously reported (Figure 1B) (Nasreen et al., 2003). Interestingly, antibody treatment did not induce ThPOK in DP thymocytes, even though they had clearly received a TCR stimulus, as evidenced by increased expression of the activation marker CD69 (Figure 1C). Hence DP thymocytes are either not susceptible to TCR-mediated ThPOK induction, or the response is delayed until transition to the $CD4^+8^{lo}$ stage.

If ThPOK expression in $CD4^+8^{lo}$ thymocytes requires ongoing TCR stimulation, then interrupting this signal should lead to termination of ThPOK expression. This was tested with $CD4^+8^{lo}$ thymocytes from $B2m^{-/-}$ $Zbtb7b^{HD/HD}$ mice, which are undergoing positive selection by class II ligands and exhibit strong ThPOK induction (Figure 1D, top panel) but cannot—because of the lack of functional ThPOK—undergo commitment to the CD4 lineage; as a result, ThPOK is only transiently induced. When TCR signaling was interrupted by placing sorted $CD4^+8^{lo}$ cells from these mice in suspension culture overnight, ThPOK transcripts disappeared, consistent with the notion that ThPOK expression required continued TCR signaling (Figure 1D). Cultured $CD4^+8^{lo}$ cells remained viable and eventually developed into SP CD8 cells (Figure 1E). If cessation of ThPOK expression in culture was caused by interruption of TCR signaling, then supplying a TCR stimulus in vitro might restore ThPOK expression. Indeed, stimulation of cultured thymocytes with anti-CD3 partly restored ThPOK expression (Figure 1D). Incomplete restoration could indicate that some cells are already too advanced toward CD8 commitment to respond to TCR stimulation, or that full induction requires additional signals not available in vitro. Sorted

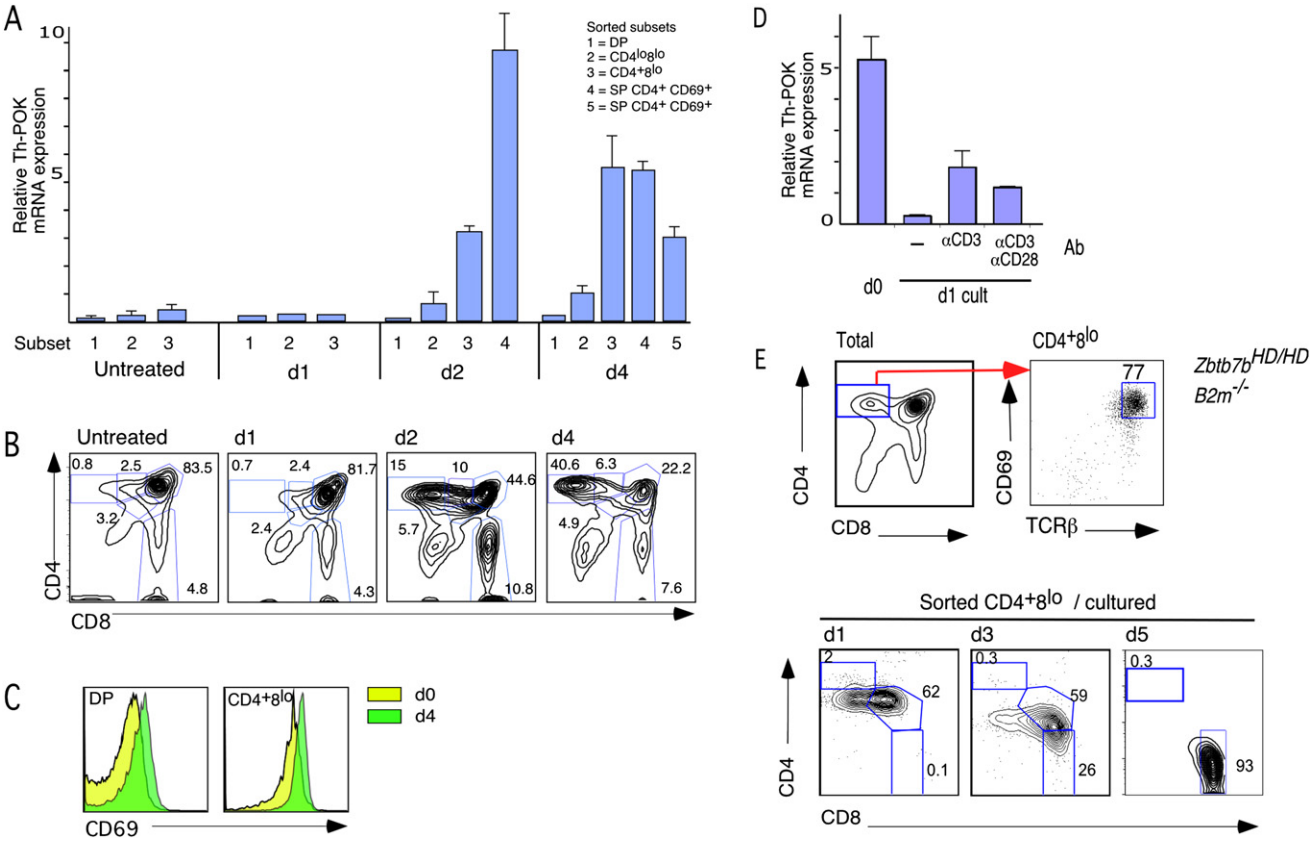

Figure 1. Strong TCR Stimulus Induces ThPOK mRNA Expression in $CD4^+8^{lo}$ Thymocytes

MHC class II-deficient mice were injected with TCRβ antibody, and analyzed at indicated time points. Shown are (A) real-time RT-PCR analysis of ThPOK mRNA expression for indicated sorted thymocyte subsets. The graphs represent mean ± standard deviation (SD) (n = 2), (B) CD4 and CD8 expression pattern of total thymocytes at indicated time points, and (C) a comparison of CD69 expression in gated DP and $CD4^+8^{lo}$ subsets before or 4 days after antibody treatment. (D) Sorted $CD4^+8^{lo}$ thymocytes from *B2m*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ mice were incubated overnight in the presence or absence of indicated antibodies, and real-time RT-PCR analysis of ThPOK mRNA was performed on indicated populations. The graphs represent mean ± SD (n = 2). (E) CD4 and CD8 expression pattern of total *B2m*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ thymocytes and sort gate used to isolate $CD4^+8^{lo}$ subset is shown. Sorted cells were also stained with anti-TCRβ and anti-CD69. Bottom FACS plots compare CD4 and CD8 expression profiles of cultured $CD4^+8^{lo}$ cells after indicated times in culture (in absence of anti-CD3 stimulation). Numbers in the FACS plot represent percentages of the associated gate. Each experiment was repeated at least three times with similar results.

$CD4^+8^{lo}$ cells from *B2m*$^{-/-}$ *Zbtb7b*$^{+/HD}$ mice maintained ThPOK expression after overnight culture, indicating that most of these cells are committed to the CD4 lineage, leading to permanent activation of the ThPOK locus. A possible alternative explanation for the specific loss of ThPOK transcripts in cultured *B2m*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ thymocytes is that sustained ThPOK induction involves a ThPOK-mediated positive-feedback loop that is blocked in the absence of functional ThPOK. However, the fact that freshly isolated $CD4^+8^{lo}$ cells from *B2m*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ and *B2m*$^{-/-}$ *Zbtb7b*$^{+/HD}$ mice expressed similar amounts of ThPOK transcripts argues against such a feedback loop (data not shown).

To test whether TCR signaling was also required for maintenance of ThPOK expression after CD4 commitment, we isolated SP CD4 thymocytes from antibody stimulated class II-deficient mice 12 days after anti-TCR treatment, when circulating antibody is expected to have largely disappeared. Nevertheless, ThPOK expression remained high in SP CD4 thymocytes (Figure S1A available online). Similarly, when class I-restricted thymocytes were redirected to the CD4 lineage by constitutive expression of ThPOK (He et al., 2005), the resulting SP CD4 thymocytes expressed endogenous ThPOK transcripts, even though their TCRs are class I specific (data not shown). Conversely, when class II-restricted thymocytes were redirected to the CD8 lineage in *B2m*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ mice, the resulting SP CD8 thymocytes failed to express ThPOK, even in the presence of a constitutive CD4 transgene that permits efficient signaling by their class II-restricted TCRs (Figure S1B). Together these results suggest that ThPOK expression in SP cells is regulated by TCR-independent mechanisms.

Defining the Minimal Genomic Region Required for Correct Lineage-Specific ThPOK Expression

ThPOK expression during thymic development is regulated at the level of mRNA, suggesting a transcriptional control mechanism. To identify the responsible *cis* elements, we first sought to define the minimal genomic region required for normal ThPOK regulation. For this purpose, BAC transgenes encompassing the ThPOK locus were crossed to the *Zbtb7b*$^{HD/HD}$ background, to determine whether CD4 development was restored, and to the

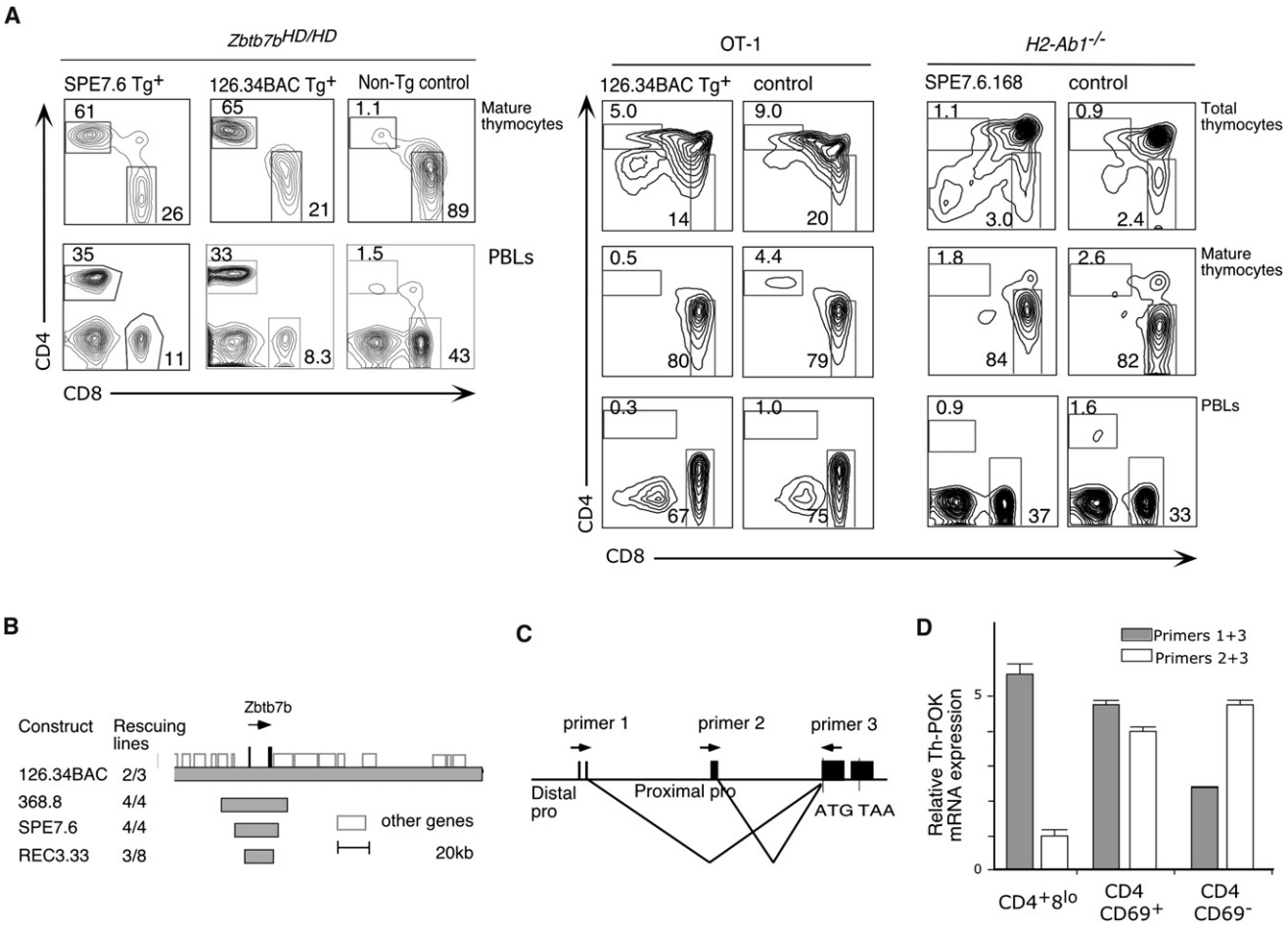

Figure 2. Minimal ThPOK Genomic Fragment Necessary for Normal Regulation of Lineage Commitment Comprises 26 kb and Encodes Two Alternate Promoters with Different Stage-Specificity

(A) CD4 and CD8 expression pattern of gated mature ($TCR^{hi}CD69^{-}$) thymocytes and PBLs from ThPOK BAC transgenic mice on the *Zbtb7b*$^{HD/HD}$ background, and similar analysis of thymocytes and PBLs from BAC transgenic mice crossed to the class I-restricted OT-1 TCR transgenic or to MHC class II-deficient mice.

(B) Schematic representation of BAC transgenic constructs, showing location of *Zbtb7b* gene. The number of founders that showed transgene expression as well as total founders is indicated.

(C) Schematic of *Zbtb7b* locus, showing positions of exons and splicing patterns of transcripts produced from alternate distal and proximal promoters.

(D) Real-time RT PCR analysis of sorted thymocyte subsets from *B2m*$^{-/-}$ mice, showing alternate transcripts produced from distal (dark bars) or proximal *Zbtb7b* promoters (white bars). Graphs are mean ± SD (n = 2).

IAb$^{-/-}$ or OT-1 TCR transgenic backgrounds, to determine whether MHC class I-restricted cells developed exclusively to the CD8 lineage. Both a full-length 200 kb BAC transgene, 126.34, and a 26 kb BAC fragment, SPE7.6, extending from 20 kb upstream to 3 kb downstream of the ThPOK coding region, fulfilled both of these requirements, indicating that they each contained all *cis* elements required for proper ThPOK regulation (Figures 2A and 2B; Figure S2). Further reducing the ThPOK genomic transgene to 17 kb, construct REC3.33, still rescued CD4 development on the *Zbtb7b*$^{HD/HD}$ background, but also severely impaired CD8 development, indicating that ThPOK expression was not appropriately suppressed in class I-restricted cells (Figure S2B). This suggested that a key regulatory element required for CD4 lineage-specific expression of ThPOK was disrupted in REC3.33 transgenic mice. Comparison of the SPE7.6 and REC3.33 transgenes indicates that the latter lacks a conserved 500 bp region at the 5′ end of the ThPOK locus, which may therefore be necessary for lineage-specific regulation of ThPOK transcription (Figure S2A, indicated by arrow).

Analysis of public CAGE and EST databases revealed two alternate ThPOK transcripts, initiating at different genomic positions (Carninci et al., 2006; CAGE track of mouse [mm5] genomic elements database hosted by RIKEN, Japan) (Figure 2C; data not shown). These transcripts utilize different upstream noncoding exons but the same ThPOK coding exons and are predicted to encode the same protein. A genome-wide analysis of histone modifications in human $CD4^{+}$ T cells demonstrated enrichment for H3-K4 methylation, which is strongly associated with active promoter regions, upstream of both noncoding exons, consistent with the occurrence of promoters at these positions (Barski et al., 2007; human genome histone methylation maps by A. Barski et al., hosted by NHLBI, USA). Real-time RT PCR and 5′ RACE analyses demonstrated that both distal and proximal transcripts were produced in murine thymocytes, but with different

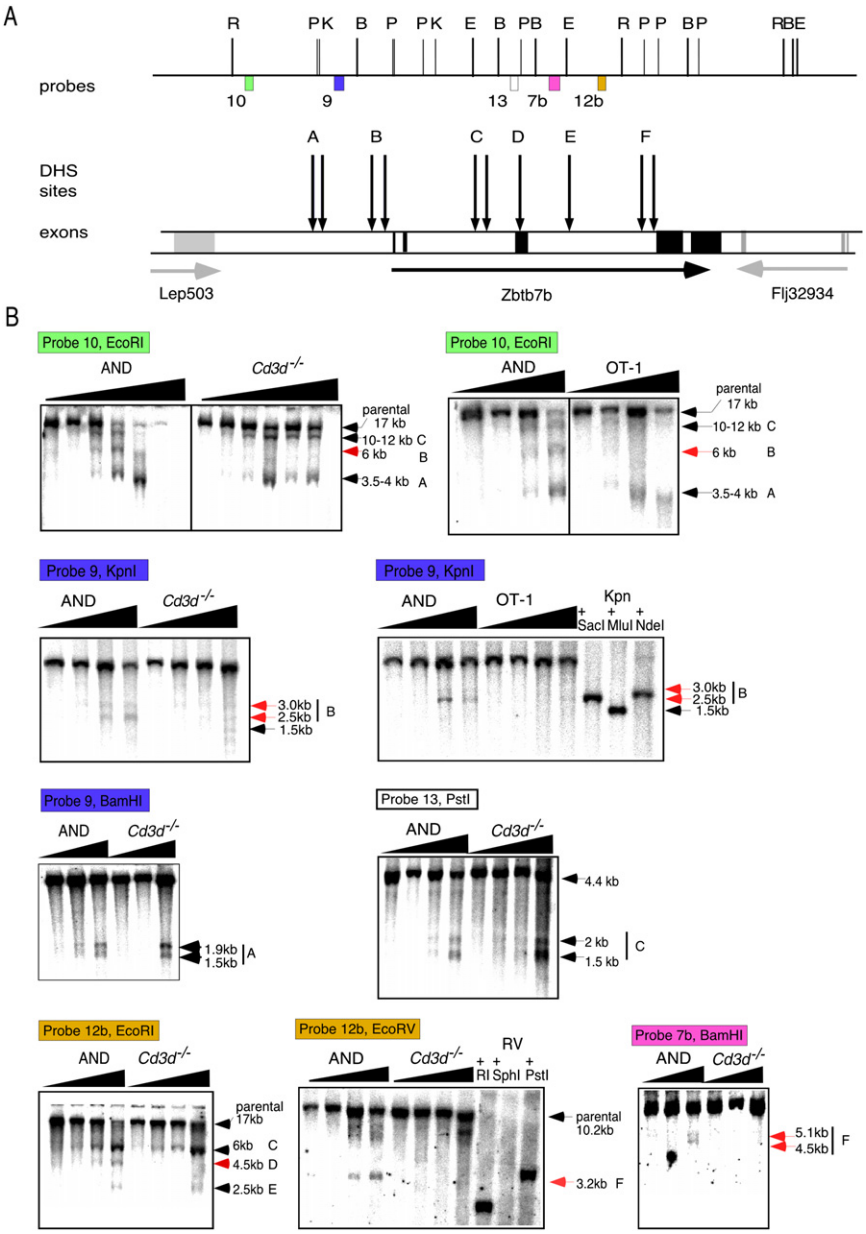

Figure 3. Multiple DHS Sites at ThPOK Locus in Primary Thymocytes Denote Potential Regulatory Elements

(A) Restriction map of ThPOK locus, showing location of probes used for DHS analysis, and position of DHS sites or clusters identified in thymocytes (R = EcoRI, p = PstI, K = KpnI, B = BamHI, E = EcoRV). (B) Representative DHS analyses using indicated probes and restriction enzymes. Analyses were carried out with total thymocytes from AND or OT-1 TCR transgenic mice, or *Cd3d*$^{-/-}$ mice. Red arrows mark DHS sites, B, D, and F, that are preferentially detected in class II-restricted thymocytes.

developmental kinetics (Figure 2D and data not shown). Thus, the distal transcript was expressed at highest amounts at the CD4^{+}8^{lo} stage, whereas maximal expression of the proximal transcript was delayed until the SP CD4 CD69^{-} stage (Figure 2D). Distal and proximal promoter usage did not correlate strictly with uncommitted or CD4-committed status. Thus proximal transcripts were detected in CD4^{+}8^{lo} cells from *B2M*$^{-/-}$ *Zbtb7b*$^{HD/HD}$ mice, which cannot undergo CD4 commitment, whereas distal transcripts were still detected in mature peripheral CD4^{+} T cells, i.e., long after CD4 commitment has occurred. The former result also shows that delayed activity of the proximal promoter does not indicate a requirement for prior ThPOK production by the distal promoter, i.e., does not reflect autoregulation of the proximal promoter by ThPOK. The fact that both promoters are utilized at the CD4^{+}8^{lo} stage before CD4 commitment suggests common regulation by a common TCR-responsive element that regulates both promoters.

Identification of Putative Regulatory Elements at the ThPOK Locus by DNase I Hypersensitive Site Analysis

DNase I Hypersensitive Site (DHS) analysis was carried out to identify potential *cis*-acting elements within the minimal 26 kb ThPOK fragment. Thymocytes from three kinds of mice were used for this analysis: (1) MHC class II-restricted AND TCR transgenic mice, in which 30%–50% of thymocytes belong to the CD4^{+}8^{lo} and SP CD4 subsets and exhibit high ThPOK expression; (2) MHC class I-restricted OT-1 TCR transgenic mice, in which thymocytes develop to the CD8 lineage and exhibit low ThPOK expression only in the CD4^{+}8^{lo} subset; and (3) *Cd3d*$^{-/-}$ mice, in which thymocytes are blocked in positive selection and lack ThPOK expression (Davé et al., 1997; data not shown). Five closely spaced probes (Figure 3A) were used for Southern analysis of DNaseI-treated genomic DNA samples from total thymocytes of these strains (Figure 3B). Six DHS sites, A–F, were identified within the region corresponding to construct SPE7.6. Site A coincided with the conserved element at the 5′ end of the ThPOK locus mentioned above, which is specifically lacking in construct REC3.33. Sites A, C, and E were present in all strains of mice examined, even *Cd3d*$^{-/-}$ thymocytes, indicating that these sites are accessible and loaded with transcription factors (TFs) prior to ThPOK transcription, although specific TFs bound may vary. These sites did not coincide with putative promoters and may instead represent enhancers or other types of regulatory elements. Sites B, D, and F were preferentially detected in AND TCR transgenic thymocytes, suggesting that they are associated with TFs only during active transcription. Sites B and D map close to distal and proximal promoters, whereas site F lies upstream of the first coding exon. DHS analysis of sorted thymocyte subsets from

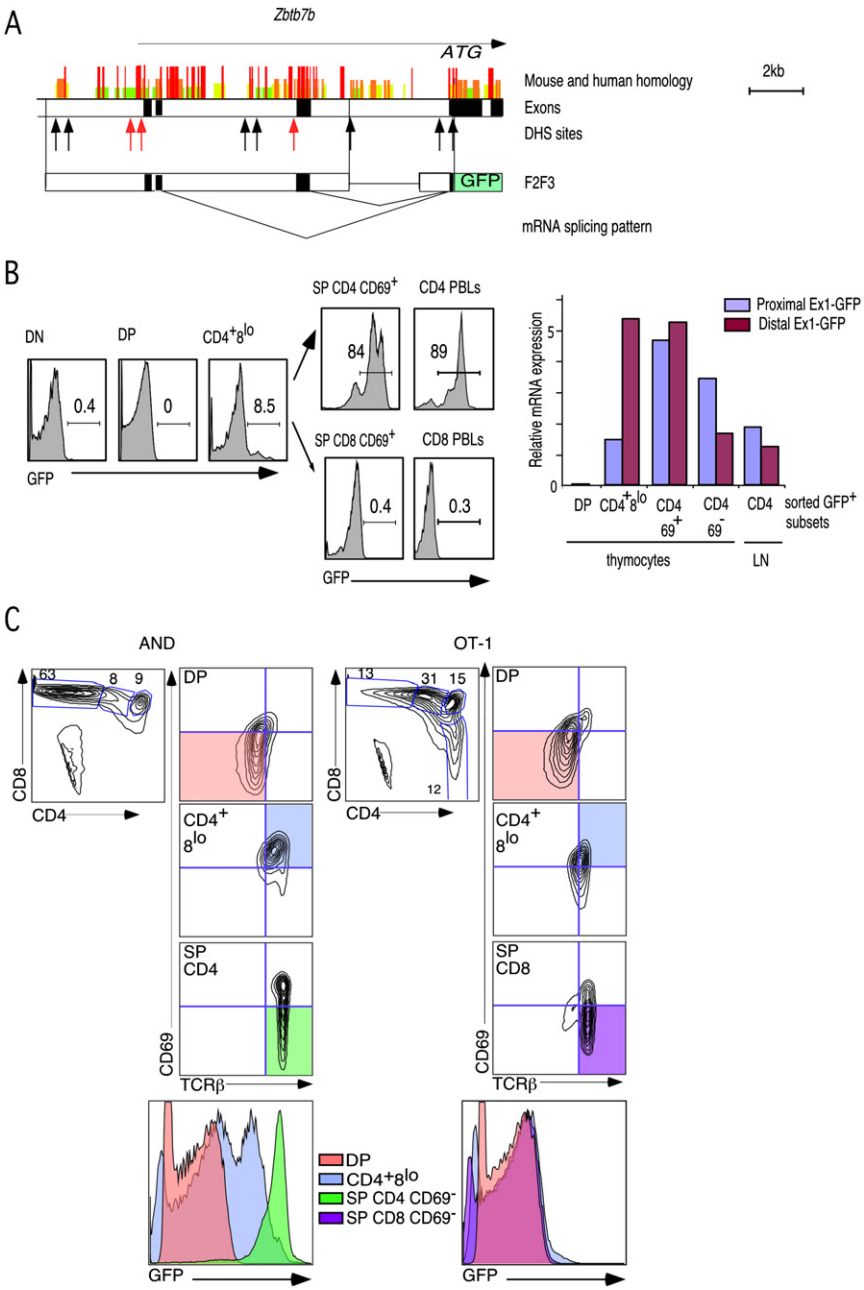

Figure 4. A 12 kb Genomic Fragment Recapitulates Normal Developmental Regulation of ThPOK Expression

(A) Top panel shows exon and intron organization of the ThPOK locus, sequence conservation between mouse and human (% homology is indicated by color; green = 78–84; yellow = 85–89; orange = 90–95; red = > 95%), position of DHS sites, and design of GFP reporter construct F2F3.

(B) Histograms of GFP reporter expression for gated thymocyte and PBL subsets, as indicated. Right panel shows real-time RT PCR analysis of GFP transcripts initiating at alternate distal or proximal ThPOK promoters in sorted F2F3 thymocyte subsets. Assays utilize specific forward primers for distal and proximal noncoding exons in combination with a common reverse GFP primer.

(C) Analysis of GFP expression in gated thymocyte subsets of F2F3 mice crossed to class I- (OT-1) or class II- (AND) restricted TCR transgenics. Top panels show gates used to define indicated subsets. Bottom panels shows GFP expression in indicated subsets.

AND TCR transgenic mice demonstrated that sites B, D, and F are lacking in preselection DP CD69^{-} thymocytes from these mice (data not shown).

Reporter Transgene Recapitulates Normal Developmental Expression Pattern of ThPOK

A GFP reporter construct, F2F3, was generated that included all DHS sites identified above, as well as both ThPOK promoters (Figure 4A). Several independent founders exhibited the same thymocyte-subset-specific expression pattern, although there was some founder-specific variation in the amount of GFP expressed and in the proportion of GFP^{+} cells (data not shown). Importantly, GFP expression in T cells was restricted to SP CD4 cells in the thymus and periphery, and some CD4^{+}8^{lo} thymocytes, recapitulating the normal expression of ThPOK (Figure 4B). Relative distal and proximal promoter usage, as detected by RT-PCR, was also similar to that of endogenous ThPOK (Figure 4B). No GFP was detected in other thymocyte subsets, or in any other peripheral lymphoid subset except CD11b^{+} monocytes, which normally also express endogenous ThPOK (data not shown). For separate examination of reporter expression in MHC class I- and class II-restricted CD4^{+}8^{lo} thymocytes, F2F3 transgenics were crossed to mice expressing the OT-1 or AND TCR transgenes, respectively. This revealed substantially higher GFP expression in class II- than class I-restricted CD4^{+}8^{lo} thymocytes (Figure 4C, bottom panels). This difference in GFP expression at the CD4^{+}8^{lo} stage, i.e., before lineage commitment, argues strongly that the F2F3 construct contains an element that mediates differential responsiveness to MHC class I- versus II-restricted TCR signals.

Identification of a Dual Silencer-Enhancer Element that Controls CD4 Lineage-Specific Expression of ThPOK

On the basis of the BAC reconstitution studies discussed above (Figure S2), we speculated that the 5′ end of the F2F3 construct marked by DHS site A might encode a CD8 lineage-specific silencer. Consistent with this hypothesis, deletion of this putative distal regulatory element (DRE), from construct F2F3 (Figure 5A, construct F2F3ΔDRE) resulted in promiscuous reporter expression in both the CD4 and CD8 lineages (Figure 5B, top). A shorter 4 kb construct that includes DHS sites C–F and the proximal

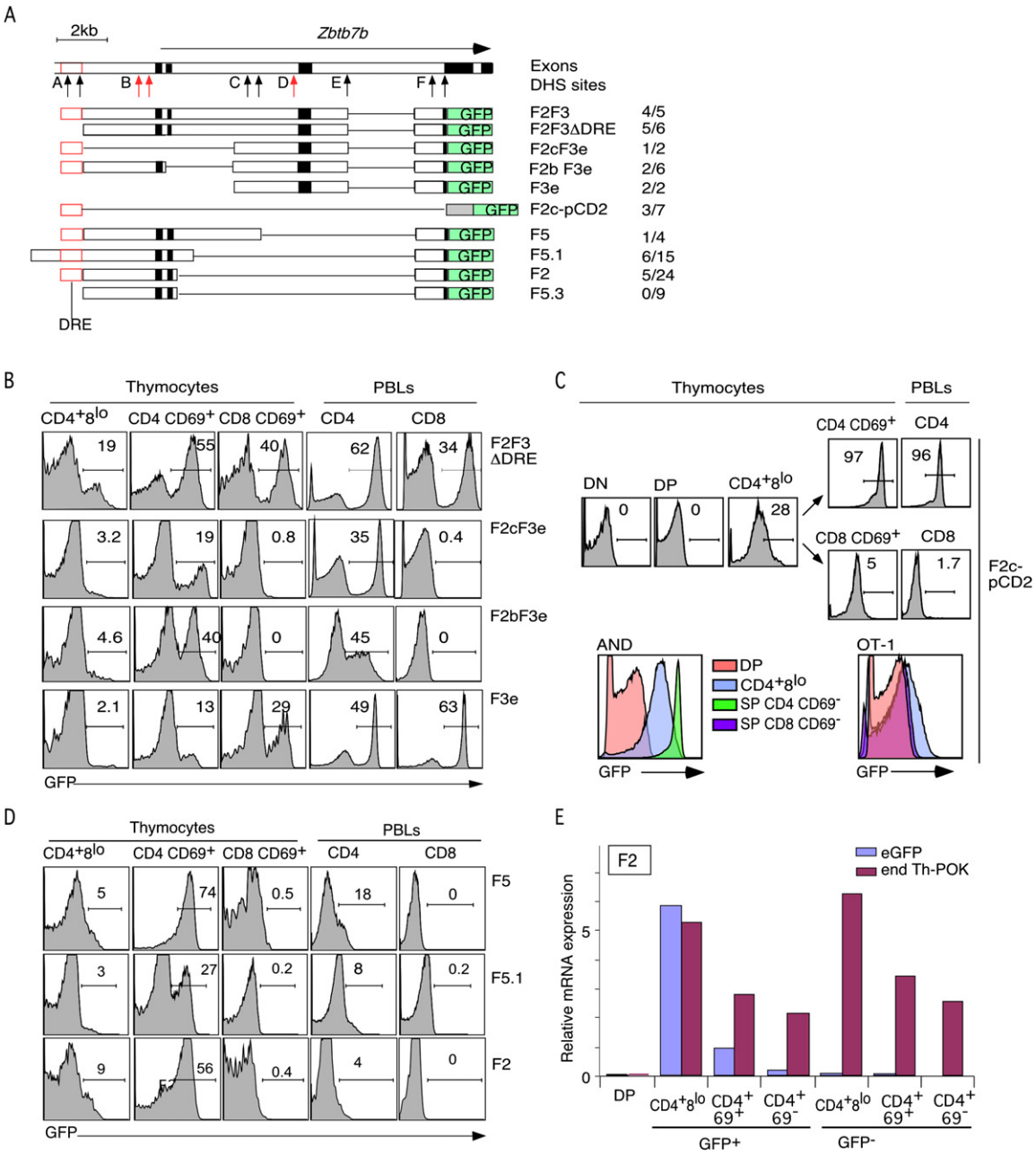

Figure 5. Identification of a Distal Regulatory Element Encoding Dual Silencer and Enhancer Functions

(A) The design of GFP reporter constructs is shown. Red, gray, and green boxes indicate positions of DRE element, minimal human CD2 promoter, and GFP reporter, respectively. The ratio next to each construct name indicates the number of founders that showed GFP expression out of total founders identified by PCR. This ratio varies between constructs, presumably in accordance with their relative susceptibility to position-dependent silencing; different founders expressing the same construct almost always exhibit the same expression pattern in CD4 versus CD8 cells, although the % of GFP$^+$ cells varies between founders (in some instances, only a single informative founder for a particular construct is available; such data are only included if they are confirmed by results from relevant closely related constructs).

(B) Histograms illustrating DRE silencer function show GFP expression for gated thymocyte and PBL subsets from indicated reporter lines.

(C) Histograms illustrating DRE enhancer function show analysis of GFP expression in F2c-pCD2 reporter line 261, on a non-TCR transgenic background (top), or crossed to class I- (OT-1) or class II- (AND) TCR transgenics.

(D) DRE mediates specific expression in class II-restricted CD4$^+$8^{lo} thymocytes in combination with the distal ThPOK promoter. Histograms of GFP expression for gated thymocyte and PBL subsets from indicated reporter lines. Data for construct F5.3, which is not expressed at any stage, is not shown.

(E) Real-time RT PCR analysis of GFP and endogenous ThPOK expression in sorted thymocyte subsets from F2 transgenic mice. Note that the GFP$^-$ CD4$^+$8^{lo} subset shows no expression of GFP mRNA, indicating a tight correlation between the onset of reporter transcription and GFP protein expression.

promoter, but excludes the DRE and the distal promoter, showed a similar promiscuous expression pattern (Figure 5A, construct F3e). Inserting the DRE in front of construct F3e restored CD4 specificity (construct F2cF3e), indicating that the DRE silencer functions independently of its distance from the promoter or enhancer elements that it regulates. Reversing the orientation of the DRE also did not affect silencer function (data not shown). Deletion of the DRE from the REC3.33 ThPOK transgene would be expected to derepress ThPOK expression in class I-restricted thymocytes, consistent with the observed phenotype of REC3.33 transgenic mice (Figure S2). We conclude that the DRE encodes a position- and orientation-independent silencer that is necessary for suppression of ThPOK transcription in the SP CD8 lineage.

For assessment of its possible enhancer function, the DRE was placed upstream of the minimal hCD2 promoter, which by itself is insufficient for transgene expression (Zhumabekov et al., 1995). Construct F2c-pCD2 mediated GFP expression in SP CD4 thymocytes and peripheral $CD4^+$ T cells (Figure 5B), indicating that the DRE also functions as an enhancer. To examine F2c-pCD2 reporter expression separately in class I- and class II-restricted thymocytes, F2c-pCD2 reporter transgenic animals were crossed to mice expressing the OT-1 or AND TCR transgenes, respectively. GFP expression was 6–10-fold higher in class II- than class I-restricted $CD4^+8^{lo}$ thymocytes. Importantly, the entire $CD4^+8^{lo}$ population in AND transgenic mice showed elevated GFP expression relative to $CD4^+8^{lo}$ cells from OT-1 mice (Figure 5C). Given that only a fraction of class II-restricted cells is committed to the CD4 lineage at the $CD4^+8^{lo}$ stage (Brugnera et al., 2000), we infer that F2c-pCD2 reporter expression in these cells precedes CD4 lineage commitment. These data indicate that the DRE element in conjunction with the heterologous CD2 promoter is sufficient for differential ThPOK expression in CD4 versus CD8-committed cells, and in uncommitted class II- versus class I-restricted $CD4^+8^{lo}$ cells. Specificity of the DRE enhancer could be inherent or might be imposed by the associated silencer.

It was important to establish whether the DRE also functions as an enhancer in the context of the endogenous ThPOK locus, in particular with respect to the distal promoter, which is the predominant promoter at the $CD4^+8^{lo}$ stage. For testing of this, four reporter constructs were generated that contained the distal promoter together with different extents of 5′ flanking DNA (Figure 5A, constructs F5, F5.1, F2, and F5.3). Analysis of corresponding transgenic mice revealed three main points: First, all constructs that included the DRE exhibited CD4 lineage-specific expression (Figure 5D). CD4 specificity requires the DRE, because a control construct in which the hCD2 enhancer is placed in front of the distal promoter instead of the DRE showed expression in both lineages (data not shown). Second, deletion of the DRE from construct F2 abrogated reporter expression (construct F5.3), indicating that the DRE enhancer is essential for expression of this construct (although other endogenous enhancers may contribute to distal promoter activity in vivo). Third, GFP expression by all three constructs was much lower in mature peripheral $CD4^+$ T cells than in SP CD4 thymocytes, in contrast to the parental F2F3 construct, which is expressed equally at both stages. At the mRNA level, reporter expression was even more restricted, i.e., essentially it was limited to $CD4^+8^{lo}$ thymocytes (Figure 5E). The discordance between mRNA and protein expression patterns reflects the longer half-life of GFP protein compared to mRNA. It is informative that GFP protein persists only in SP CD4 but not SP CD8 thymocytes, because it implies that reporter transcription at the $CD4^+8^{lo}$ stage is limited to class II-restricted thymocytes, which are the direct precursors of SP CD4 cells. Hence, the DRE enhancer in the context of the distal promoter supports mRNA expression specifically in class II-restricted $CD4^+8^{lo}$ thymocytes. Restricted expression to the $CD4^+8^{lo}$ subset appears to require the combination of the DRE with the distal ThPOK promoter, because neither the combination of the DRE with the hCD2 promoter nor the combination of the hCD2 enhancer with the distal ThPOK promoter (data not shown) shows this stage-specificity.

Identification of a Non-Lineage-Specific Enhancer at the ThPOK Locus

Deletion of the DRE from construct F2F3 did not abolish reporter expression, indicating the presence of an additional enhancer within this construct. In contrast, deletion of the DRE from the smaller F2 construct completely abolished reporter expression (construct F5.3), indicating that the additional enhancer is lacking from F2 or acts only on the proximal promoter. To map this second enhancer, a further series of reporter transgenes was generated that were centered on the proximal promoter (Figure S3A). Analysis of corresponding transgenic mice revealed three main points: First, all constructs that included the region around DHS site C exhibited expression in both CD4 and CD8 lineages (constructs F3, F3d–f). Some of these constructs additionally exhibited expression at the DP stage, in particular construct F3d (Figure S3). Second, deletion of DHS site C caused dramatic reduction in reporter expression, particularly in the CD8 lineage (constructs F3b, F3bF6), indicating that this region encoded an important positive regulatory element, designated as the "general T lymphoid element" (GTE). In contrast, deleting DHS site E did not markedly affect promiscuous reporter expression (Figure S3B, construct F3d-ΔPRE). Third, although deletion of the GTE severely diminished reporter expression, some expression remained, implying the existence of another, weaker enhancer. This remaining expression is abolished by simultaneous deletion of DHS sites C and E (construct F3b.1), indicating that another positive regulatory element, designated as the "proximal regulatory element" (PRE), maps near DHS site E. Interestingly, constructs that depend on the PRE for enhancer function exhibit preferential expression in the CD4 lineage (constructs F3b, F3bF6), although it is unclear whether this preference is dependent on the PRE, the proximal promoter, or the combination of both. Only a subset of SP CD4 thymocytes and peripheral $CD4^+$ T cells exhibited PRE-dependent reporter expression, which may mark a particular T helper subset or may reflect a high degree of random transgene silencing for these constructs. Finally, to test the functional relevance of DHS site F, located immediately upstream of the ThPOK coding exons, we modified construct F3 by inserting a reporter cDNA directly into the proximal noncoding exon, allowing the downstream splice acceptor-reporter cassette that includes DHS site F to be omitted (Figure S3B). Omission of DHS site F did not alter the reporter expression pattern for constructs F3 or F3b, indicating that it does not markedly contribute to transcription in the context of these constructs (Figure S3B, and data not shown).

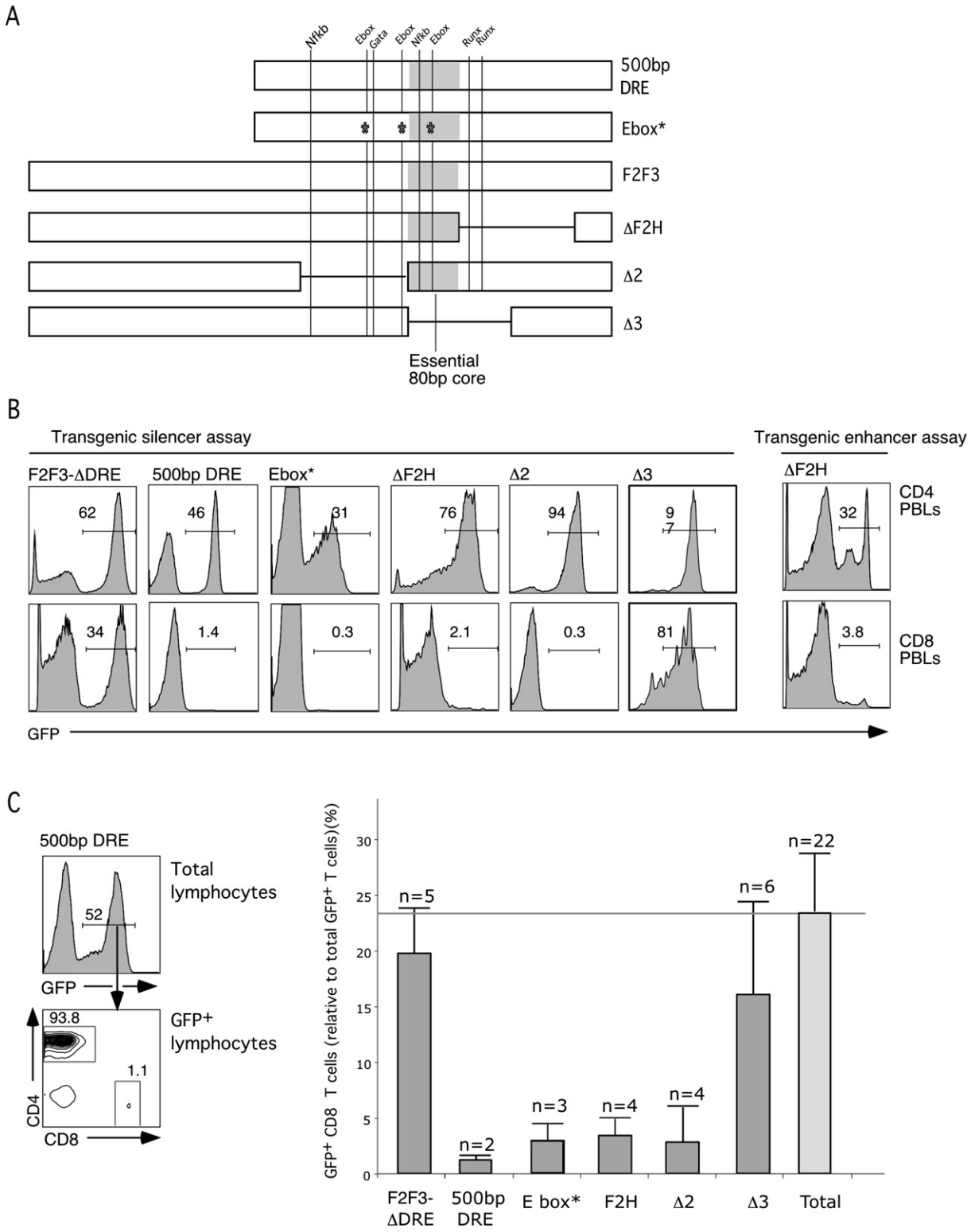

Figure 6. Mutational Dissection of DRE Element Defines 80 bp Core Element

(A) Schematic of DRE mutants indicating deletion boundaries, mutations, and positions of conserved TF consensus sites. The gray box indicates the 80 bp core region that is essential for silencer function.

(B) Histograms of GFP expression for gated SP CD4 and CD8 peripheral T cells from indicated reporter lines. Numbers in the histograms indicate % GFP$^+$ cells.

(C) The bar graph summarizes the proportion of GFP$^+$ T cells belonging to the CD8 lineage for multiple founders of each construct (in example at left % GFP$^+$ CD8 cells = 1.1/ (93.8 + 1.1) × 100% = 1.2%). For comparison, the proportion of CD8 T cells out of total peripheral T cells (23%) is indicated at the right of the bar graph.

Consistent with lack of function for DHS site F, construct F3b.1, which lacks the GTE and PRE elements but retains DHS site F, shows no expression.

Functional Dissection of the DRE Element

The above results demonstrate that the DRE is the main element responsible for specificity of ThPOK transcription during thymic development. In particular, our data suggest a mechanism whereby the DRE silencer prevents ThPOK transcription, unless it is turned off or converted to an enhancer in response to a class II-restricted TCR signal. To elucidate the pathway(s) that control this switch, it is important to identify the transacting factors (TFs) that regulate DRE function. Interspecies nucleotide comparison of the DRE indicates that a region of 300 bp is strongly conserved between placental and marsupial mammals (Figure S4A, colored regions). A number of conserved sites of potential functional relevance occur within this region, including one Gata, two Runx, two NF-κB, and three E-box consensus sites. Three deletion mutants of the DRE element were generated in order to further narrow the region under consideration: ΔF2H, Δ2, and Δ3, which each lacked about 170 bp of the conserved region (Figure 6A). For assessment of their silencing function, the variant elements were inserted upstream of reporter construct F2F3ΔDRE, which lacks a DRE element and thus exhibits promiscuous expression in both CD4 and CD8 lineages (Figure S4B), and the resulting constructs were used to generate transgenic mice. In the transgenic assay, silencing function of the variant DRE elements was measured by their ability to suppress GFP reporter expression in CD8 cells (Figures 6B and 6C). Constructs Δ2 and ΔF2H showed quite efficient suppression of GFP expression in CD8 cells, demonstrating that the deleted regions are dispensable for silencing. Of note, the Δ2 and ΔF2H deletions removed the Gata and Runx consensus motifs, respectively, indicating that neither is essential for silencer function. In contrast, construct Δ3 largely fails to repress GFP expression in CD8 cells, indicating that a central 80 bp core region that is uniquely deleted in Δ3 is primarily responsible for silencing (Figure 6A, the 80 bp core indicated in gray). Comparison of multiple Δ3 founders suggests that this construct may retain some weak partial silencing activity, so that a minor contribution to silencing by sites outside of this region cannot be excluded (Figure 6C, note that the average proportion of GFP^+ CD8 cells seems somewhat reduced in Δ3 compared to ΔDRE mice). By inserting the ΔF2H DRE variant in front of the hCD2 promoter (construct F2cΔF2H-pCD2), which lacks its own enhancer, we further demonstrate that Runx sites were also not required for enhancer activity of the DRE element (Figure 6B). The 80 bp core region required for silencer function contains both an NFκB and E-box motif. E-box motifs could mediate regulation by E proteins such as E2A and HEB whose activity is known to be controlled by TCR-mediated signals. However, mutation of all three E-box motifs within the DRE element does not markedly impair silencing activity (Figures 6B and 6C). A functional role for the NFκB site cannot be excluded at this point, although previous analyses of knockout mice affecting this pathway do not support a role in CD4-CD8 lineage commitment. Hence, it appears likely that the DRE silencer is regulated by a factor (or factors) whose role in lineage commitment is not yet recognized.

DISCUSSION

ThPOK induction represents the earliest available indicator of CD4 commitment, thus providing a valuable new access point from which to elucidate the responsible upstream pathways. The present study contributes to this goal in two respects. First, we provide compelling evidence of mechanistic linkage between TCR signaling and ThPOK induction. Second, we demonstrate that ThPOK induction is controlled at the level of transcription and identify a key element, the distal regulatory element, that mediates selective expression in MHC class II-restricted thymocytes. The DRE encodes dual silencer and enhancer functions, thus making it an ideal molecular switch. We propose a mechanism whereby THPOK transcription and hence CD4 commitment depend on overcoming DRE silencer function.

Our observations that in vivo antibody-mediated TCR treatment leads to ThPOK induction in $CD4^+8^{lo}$ thymocytes and that $CD4^+8^{lo}$ thymocytes require TCR stimulation for continued ThPOK expression in vitro together provide strong support for a causal relationship between TCR engagement and ThPOK induction. The simplest and most plausible interpretation of these experiments is that TCR stimulation of $CD4^+8^{lo}$ thymocytes directly triggers ThPOK transcription in those cells. The observation that ThPOK is not induced until the $CD4^+8^{lo}$ stage is most consistent with the kinetic signaling model of lineage commitment, and less so with the instructional model, which postulates that lineage-determining TCR signals are transmitted at the DP stage. Nevertheless there are two important caveats to this interpretation. First, ThPOK induction at the $CD4^+8^{lo}$ stage could represent a delayed response to TCR signals received at the DP stage, which might, for instance, initiate changes in chromatin conformation that are essential for later ThPOK induction. Second, we cannot exclude the possibility that additional stimuli are required for CD4 commitment. Definitive resolution of these issues will require elucidation of the upstream pathways that control ThPOK induction.

We have identified several important *cis* elements at the ThPOK locus that control its differential expression in class I- versus class II-restricted thymocytes. The ThPOK locus is marked by a dense concentration of conserved noncoding DNA regions, many of which are preserved even between placental and marsupial mammals, implying important regulatory functions. Six distinct DHS sites and clusters are accessible in thymocytes and map within or near these conserved regions. Two DHS sites, B and D, correlate with ThPOK transcription and mark the distal and proximal promoters, respectively, whereas three constitutive sites, A, C, and E, correspond to distinct DRE, GTE, and PRE regulatory elements, respectively. Each of the latter elements acts as an enhancer but with different lineage specificity. The PRE in the context of the proximal promoter shows partial preference for the CD4 lineage, although this

If the proportion of GFP^+ CD8 cells out of the total GFP^+ T cells is the same as this value, it indicates that silencing is abolished, whereas if it is significantly lower, silencing is maintained. Graphs are mean ± SD (n is indicated above each bar).

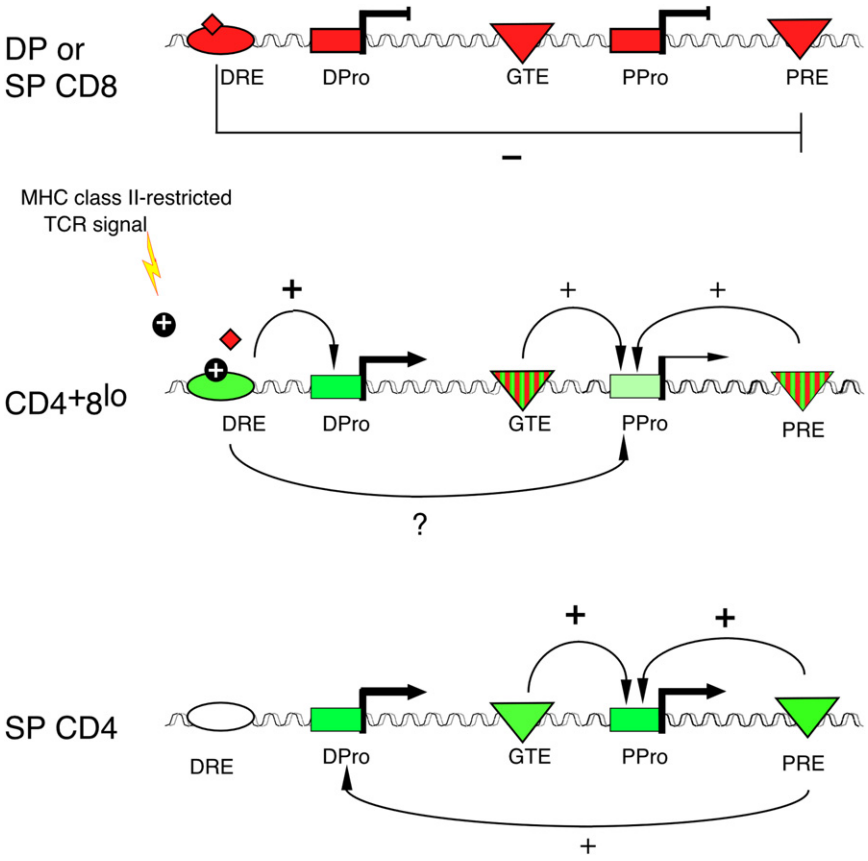

Figure 7. Model of ThPOK Transcriptional Control

Schematic representation of the activity of ThPOK *cis* elements at different thymocyte stages, as indicated. Approximate positions of promoters, enhancers, and the DRE element are indicated by boxes, triangles, and ovals, respectively. Red, green, and striped shading of elements indicates that they are repressed by the DRE silencer, or exhibit full and partial activity, respectively. Strong or weak promoter activity is indicated by thick or thin arrows. Arrows extending from one *cis* element to another indicate functional interactions, whereby + and − symbols denote positive or negative control.

preference may require cooperation with the proximal promoter. The GTE in the context of the proximal promoter shows promiscuous activity in both CD4 and CD8 lineages. Finally, the DRE in the context of the distal promoter or the heterologous hCD2 promoter is active only in class II-restricted thymocytes.

We propose that the DRE is the primary *cis* element at the ThPOK locus responsible for receiving and translating upstream lineage-specifying signals, for the following reasons: The DRE mediates exclusive expression in class II-restricted thymocytes both in combination with the endogenous distal promoter and the heterologous hCD2 promoter. The latter result indicates that specificity to class II-restricted thymocytes is encoded by the DRE rather than the distal promoter. The DRE mediates expression at the $CD4^+8^{lo}$ stage, the stage at which lineage commitment may be decided. In fact, in the context of the distal promoter, the DRE mediates transcription exclusively at this stage. The DRE imposes specificity for class II-restricted cells on other *cis* elements that lack this inherent capacity, notably the GTE element. Finally, our studies have identified no other element that confers specificity to class II-restricted cells and is also active at the $CD4^+8^{lo}$ stage. Although the PRE mediates preferential expression in the CD4 lineage, it supports little to no expression at the critical $CD4^+8^{lo}$ stage and may be more relevant for post-CD4-commitment expression.

A striking feature of the DRE is that it functions as both a silencer and an enhancer. Silencer function is essential for enforcing CD4 lineage-specific transcription and for proper regulation of lineage commitment. Whether or not the enhancer function of the DRE is also essential for lineage commitment is unknown and will be difficult to assess unless these functions can be separated. If these functions are, in fact, inseparable, it would indicate that the same regulatory site controls both silencer and enhancer activities, and that the choice between them depends on competition between silencing and activating factors for binding to this site. A key unresolved question is the identity of the factors that control DRE activity. On the basis of our mutational analysis, we can rule out direct regulation of the DRE element by at least two potential suspects, i.e., Gata-3 and Runx3. However, this does not exclude indirect regulation of DRE activity by one of these factors. Studies to identify the factors that directly control DRE activity are currently underway employing affinity purification and other approaches.

We propose the following model for transcriptional control of ThPOK during thymic development (Figure 7): At the DP stage, ThPOK transcription is actively repressed by a DRE-dependent mechanism, consistent with induction of ThPOK when the DRE is deleted. At the $CD4^+8^{lo}$ stage, strong TCR signaling mediated by class II ligands activates or induces a transacting factor that converts the DRE from silencer to enhancer mode and initiates ThPOK transcription. Alternatively, TCR-mediated derepression of the DRE silencer may be initiated at the DP stage but not completed until the $CD4^+8^{lo}$ stage. In SP CD4 thymocytes and peripheral $CD4^+$ T cells, ThPOK transcription is maintained by a TCR-independent mechanism. This seems to require the GTE and PRE elements, because the DRE loses the ability to act as an enhancer at the SP CD4 stage, at least in the context of the distal promoter. Inactivation of the ThPOK locus in SP CD8 thymocytes and peripheral $CD8^+$ T cells requires the DRE, at least for initiation but perhaps not for permanent maintenance of silencing. In this model, other regulatory elements at the ThPOK locus, including enhancers and promoters, are not targets of TCR signaling but become active by default when DRE silencer activity is blocked. Although these elements are not involved in initiating CD4 commitment, they are probably

important in achieving the appropriate level of ThPOK expression required to complete CD4 commitment. Whether any elements other than the DRE are individually indispensable for CD4 commitment remains to be established.

EXPERIMENTAL PROCEDURES

Mice

CD4 (Killeen and Littman, 1993) (kindly provided by S. Reiner) and OT-1-TCR (Hogquist et al., 1994) transgenic lines, as well as Zbtb7b$^{-/-}$ (Davé et al., 1998), MHC *IAb*$^{-/-}$, and *Cd3d*$^{-/-}$ (Davé et al., 1997) mice, have been described previously. All other transgenic lines described in this paper were generated by the FCCC Transgenic Facility. Animal care was in accordance with National Institutes of Health (NIH) guidelines.

TCR Stimulation Assays

For in vivo α-TCR stimulation, young adult IAβ$^{-/-}$ mice were injected intraperitoneally with 30 μg of α-TCRβ antibody (H57) per 1 g of body weight. For in vitro stimulation, 10^6 sorted thymocytes were cultured in DMEM with 10% FSC on 96-well plates precoated with 20 μg/ml each of α-CD3 (2C11) and α-CD28.

Transgenic Constructs

BAC constructs 368.8, SPE7, and REC3.33 were derived form BAC clone 368D24 (C57BL/6 RPCI-23 BAC library, Genome Sequence Centre, BC Cancer Agency) by restriction digestion or recombineering, according to established protocols (see the recombineering web site hosted by NCI-Frederick). To generate GFP reporter constructs, we first generated a splice acceptor cassette, consisting of an EGFP cDNA (pEGFP-N1, Clontech) inserted into the first ThPOK coding exon in the context of a 1.3 kb genomic fragment (this extends from 1.3 kb upstream of the first ThPOK coding exon to the ATG start codon and includes the splice acceptor site). Final reporter constructs were generated by inserting the desired ThPOK genomic fragments into a HindIII site upstream of this splice acceptor cassette. For construct F2cpCD2, the EGFP cassette was inserted downstream of the minimal hCD2 promoter (Zhumabekov et al., 1995). For constructs utilizing the dsRed reporter (pDsRedT.4.str, kind gift of Ben Glick), the reporter cDNA was inserted into the proximal ThPOK noncoding exon (constructs F3-dsRed and F3dΔPRE-dsRed). Precise sequences of all constructs are provided in supplementary materials.

Flow Cytometry

Cells were prepared from thymus and peripheral blood and analyzed by flow cytometry according to standard procedures. All antibodies were obtained from BD-PharMingen.

Real Time RT-PCR

Real-time RT PCR analysis for Th-POK and EGFP was carried out according to the probe-based method and analyzed by the comparative Ct method (compared to β-actin). Primer and probe sequences are provided in the Supplemental Data.

DNase I Hypersensitivity Analysis

DHS analysis was carried out according to standard procedures. In brief, 10^7–10^8 primary thymocytes were allowed to swell in ice-cold high-salt buffer (10 mM Tris pH 7.4, 100 mM NaCl, 30 mM MgCl2, 0.1% NP-40) for 15 min, and nuclei were isolated by centrifugation. The nuclear pellet was then resuspended in low-salt buffer (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM MgCl2, 0.1% NP-40 buffer), divided into 120 μl aliquots, and briefly treated with varying concentrations of DNase I (Worthington: DPRF). DNase I digestion was stopped by addition of an equal volume of cell lysis buffer and DNA was isolated with Genomic DNA purification kit (Puregene). Southern blotting was carried out according to standard procedures with different probes, as indicated in Figure 3. Probe sequences are provided in the Supplemental Data.

SUPPLEMENTAL DATA

Additional Experimental Procedures and four figures are available at http://www.immunity.com/cgi/content/full/28/3/346/DC1/.

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